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14. ABSTRACT

The objective of this study is to understand the biological determinants of breast density, a strong predictor of human breast cancer risk, in an animal model of in utero insulin-like growth factor-1 (IGF-1) exposure. In utero exposure of 5 µg of total IGF-1 during gestational days 10 to 18 in pregnant wild type C57BL/6J or IGF-1 deficient mice resulted in significantly heavier body weights of the offspring at post-natal day 3, day 7, and even up to day 21 when compared to PBS controls, but not at birth. This positive association was extended to levels of putative breast stem/progenitor CD49f14. ABSTRACT +CD24+ and CD49f+CD29 +CD24+ cells and breast density, as assessed by the sum of the different mammary gland structures per unit area per gland. The findings demonstrate that in utero exposure to low levels of mitogens such as IGF-1 may be sufficient to influence cancer risk in the adult life, in particular breast cancer.

15. SUBJECT TERMS
Breast stem cells, breast density, insulin-like growth factor-1, perinatal factors, cancer risk factors

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INTRODUCTION

The objective of this proposed study is to understand the biological determinants of mammographic density, a strong predictor of human breast cancer risk. Our hypothesis is that the *in utero* levels of mitogens, such as insulin-like growth factor-1 (IGF-1), drive an increased number of breast stem/progenitor cells in the mammary gland, which in turn results in a more extensive epithelial mammary tree formation, i.e., an increase in mammographic density. Since breast stem/progenitor cells are the cell population within the mammary gland that is presumed to be susceptible to malignant transformations, dense breast with elevated levels of stem/progenitor cells should have a higher risk of becoming malignant. The study hopes to correlate *in utero* levels of IGF-1 administered to the mother with the birth weight, breast/mammographic density and number of breast stem/progenitor cells of the offspring.

BODY

Timetable of research accomplishments as outlined in the Statement of Work:

a. Obtain approval for animal research; develop and validate microparticle delivery vehicle generation. (Months 0-3)
b. Obtain IGF-$1^{m/m}$ and control mice and begin breeding. (Months 3-6)
c. Perform preliminary studies to quantitate and validate breast stem/progenitor FACS assay and breast density assay. (Months 3-6)
d. Perform experiments on effect of *in utero* IGF-1 treatment on breast stem/progenitor pools and breast density using cohorts of bred mice. (Months 8-32)
e. Compile data, perform statistical analysis and write manuscript. (Months 32-36)

Final progress report:

Since the launching of the project, we have obtained approvals for animal research from the Institutional Animal Care and Use Committee (IACUC), University of Massachusetts Medical School (UMMS) in May 2008 and from the Animal Care and Use Review Office (ACURO), Department of The Army in June 2008 (Task a). We submitted a minor amendment and obtained approval to deliver IGF-1 by intraperitoneal (ip) injections instead of by microparticle delivery involving surgery (Task a).

Homozygous *Igf1*$^{m/m}$ mice were obtained from Jackson Laboratory and bred for the project (Task b). While this was being carried out, preliminary studies were carried out using C57BL/6J wild type mice (Task c). First, we performed preliminary studies to quantitate and validate breast density by histological assays and *in vivo* and explant imaging. Histologically, we stained mammary glands from virgin and pregnant C57BL/6J mice using whole-mount staining protocols as reported by Nandi (1958), Fata et al., (1999) and Brisken et al., (2002). Although all three methods gave good staining of mouse mammary glands as visualized under a microscope, the method as reported by Brisken gave the best staining of terminal end buds (Figure 1). We improved on the method by cover-slipping the slides using Permount whereby the stained mammary glands are preserved indefinitely and can be viewed microscopically for analysis at any time.

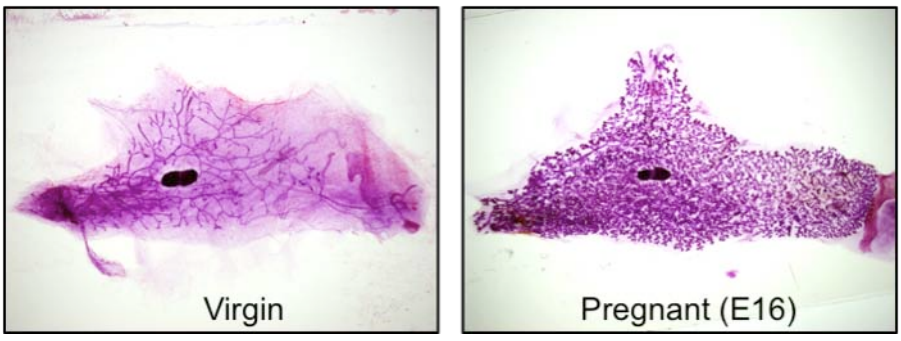

Figure 1. Whole mount fourth inguinal mammary glands of C57BL/6J mice stained with carmine alum solution, mounted and cover-slipped on glass slides using Permount showed that a mammary gland from a virgin mouse (left panel) is “less dense” than that of a E16 pregnant (right panel); both glands are from 8 week-old mice.

Second, we performed preliminary studies to quantitate and validate breast stem/progenitor cells using flow cytometric assay (Task c). Using protocols adapted from StemCell Technologies Inc. (Vancouver, Canada) and Stingl et al., (2006), we were successful in detecting putative breast stem/progenitor sub-populations from

mammary glands that are CD49f$^+$ and CD24$^+$ (Figure 2); CD49f$^+$, CD24$^+$, and CD29$^+$ (Visvader and Lindeman, 2006); CD29$^+$ and CD24$^+$ (Shackleton et al., 2006); and CD49f$^+$ and EpCAM$^+$ (Stingl et al., 2001).

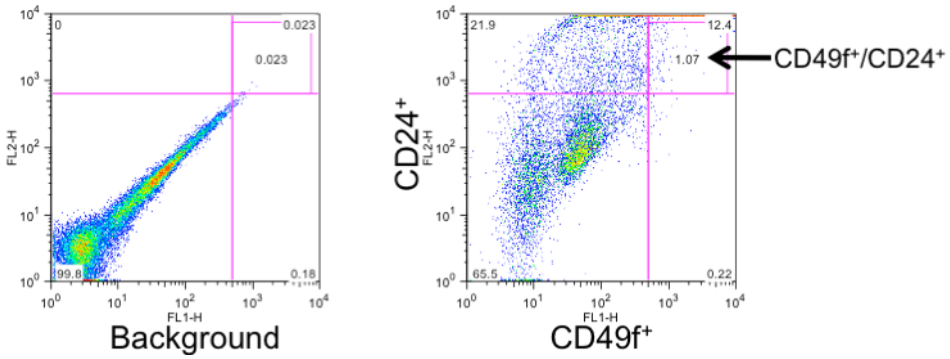

Figure 2. Flow cytometric dot plots showing the identification of a population of CD49f$^+$CD24$^+$ cells in the upper right quadrant (arrow) from C57BL/6J dissociated mammary cells.

We have successfully established an *in utero* model for the effect of IGF-1 on body weight, putative breast stem/progenitor cells, and breast density of the offspring (Task d). Daily ip injections of IGF-1 were more effective when administered from gestational day (GD) 10 to 18 than from GD 10 to 16. We investigated the prenatal effect of delivering a total amount of 2.5, 5, 10, 20, and 50 µg IGF-1 to pregnant wild type mice, with equal volumes of phosphate-buffered saline (PBS) administered to control animals.

In four independent experiments on C57BL/6J mice, we found that the *in utero* administration by ip injections of a total amount of 2.5, 5, 10, 20, and 50 µg of IGF-1 per animal from GD 10 to 18 did not result in any significant change in the birth weight (postnatal day 1, P1) of the pups when compared to PBS controls. However, the administration of 5 µg of IGF-1 resulted in significantly heavier body weights of the pups at P3; $p = 0.001$; *t*-test), P7 ($p < 0.0001$; *t*-test), and even up to P21 ($p = 0.002$; *t*-test) when compared to PBS controls (Figure 3A). The significantly heavier body weight at P21 by the treatment of 5 µg of IGF-1 was contributed by both female and male offspring (Figure 3B) with additional significant effects contributed by female offspring only at 10 and 20 µg of IGF-1 when compared to PBS controls.

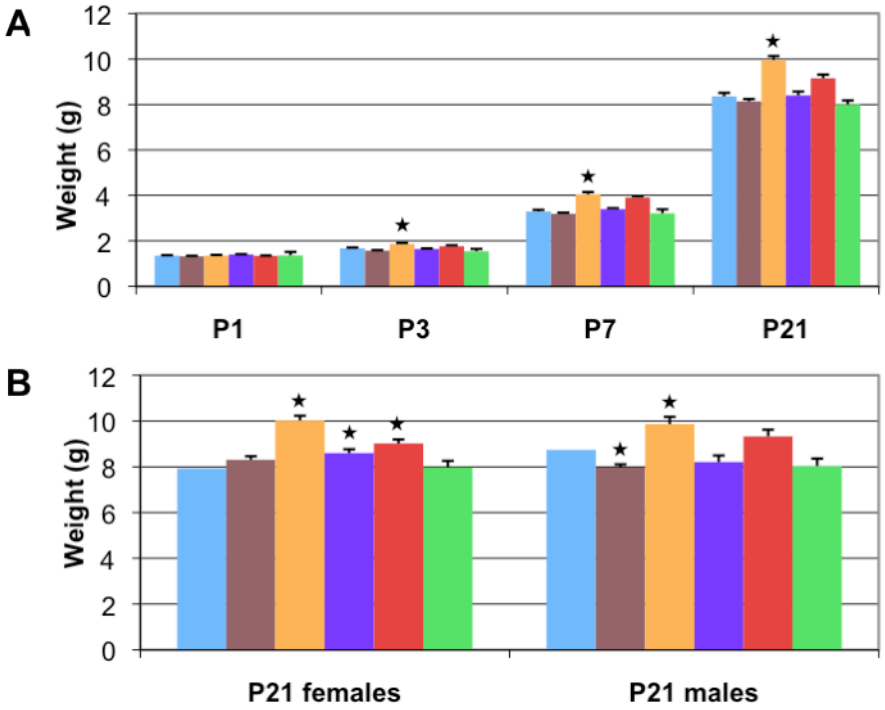

Figure 3. (A) Histograms showing the mean body weights of female and male C57BL/6J offspring at post-natal day 1 (P1), P3, P7, and P21 born to mothers that were injected with PBS (blue, n = 38 - 40) or IGF-1 consisting of 2.5 µg (brown, n = 27), 5 µg (yellow, n = 21), 10 µg (purple, n = 27 – 28), 20 µg (red, n = 18 - 20), or 50 µg (green, n = 20). (B)

Histograms showing the mean body weights of female and male offspring separately at P21. Error bars represent standard error of the mean (SEM). * denotes significance of $p < 0.05$ by *t*-test when compared to PBS controls.

Female pups were sacrificed between P29 and P31 and their left fourth inguinal mammary glands were enzymatically dissociated into single cells for the detection of putative breast stem/progenitor cell markers by flow cytometry. Of the putative markers of breast stem/progenitor cells analyzed, including the $CD49f^+CD24^+$ (Stingl et al., 2006a), $CD29^+CD24^+$ (Shackleton et al, 2006), $CD49f^+CD29^+CD24^+$ (Visvader and Lindeman, 2006), and $CD49f^+EpCAM^+$ (Stingl et al., 2001) subpopulations, only treatment with 5 µg of IGF-1 resulted in a significant increase in the $CD49f^+CD24^+$ and $CD49f^+CD29^+CD24^+$ subpopulations ($p = 0.03$ and 0.04, respectively; *t*-test) when compared to PBS controls (Figure 4). However, there was no significant difference in the number of $CD29^+CD24^+$ and $CD49f^+EpCAM^+$ cells for all treatment groups when compared to controls (Figure 4).

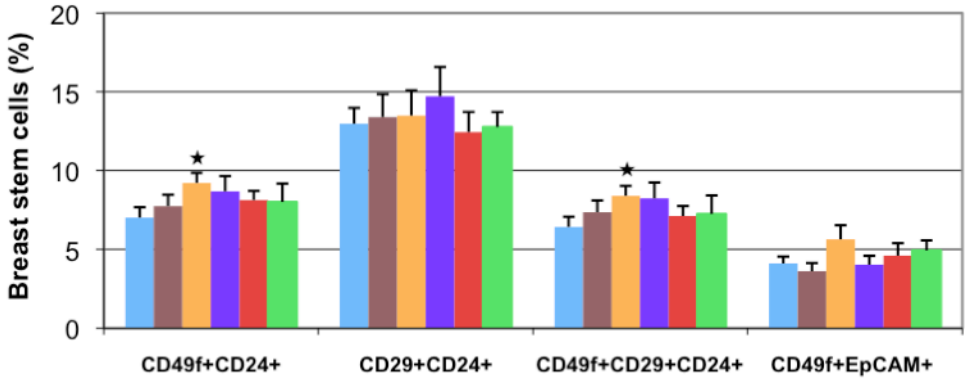

Figure 4. Histograms showing the percentages of putative breast stem/progenitor cell populations from mammary glands of C57BL/6J female offspring born to mothers that were injected with PBS (blue, n = 15) or IGF-1 consisting of 2.5 µg (brown, n = 9), 5 µg (yellow, n = 11), 10 µg (purple, n = 10), 20 µg (red, n = 10), or 50 µg (green, n = 11). Error bars represent standard error of the mean (SEM). * denotes significance of $p < 0.05$ by *t*-test when compared to PBS controls.

Contra-lateral fourth inguinal mammary glands of the same pups that were used for breast stem/progenitor cells detection were subjected to whole mount staining with carmine alum solution. First, morphometric analyses showed that IGF-1 treatments of 5 µg resulted in longer duct elongation when compared to controls (Figure 5). Additionally, among the different mammary gland structures identified (Figure 6A), treatment with 5 µg of IGF-1 resulted in significantly higher numbers of lateral buds (LB), ducts (D), and criss-crosses (CC) when compared to controls ($p = 0.01$, 0.0005, and 0.05, respectively, *t*-test) (Figure 6B). Using the sum of all the mammary structures per unit area per gland as a measure of breast density, we found that the *in utero* treatment with 5 µg of IGF-1 resulted in the densest mammary gland ($p = 0.002$; *t*-test) (Figure 6C).

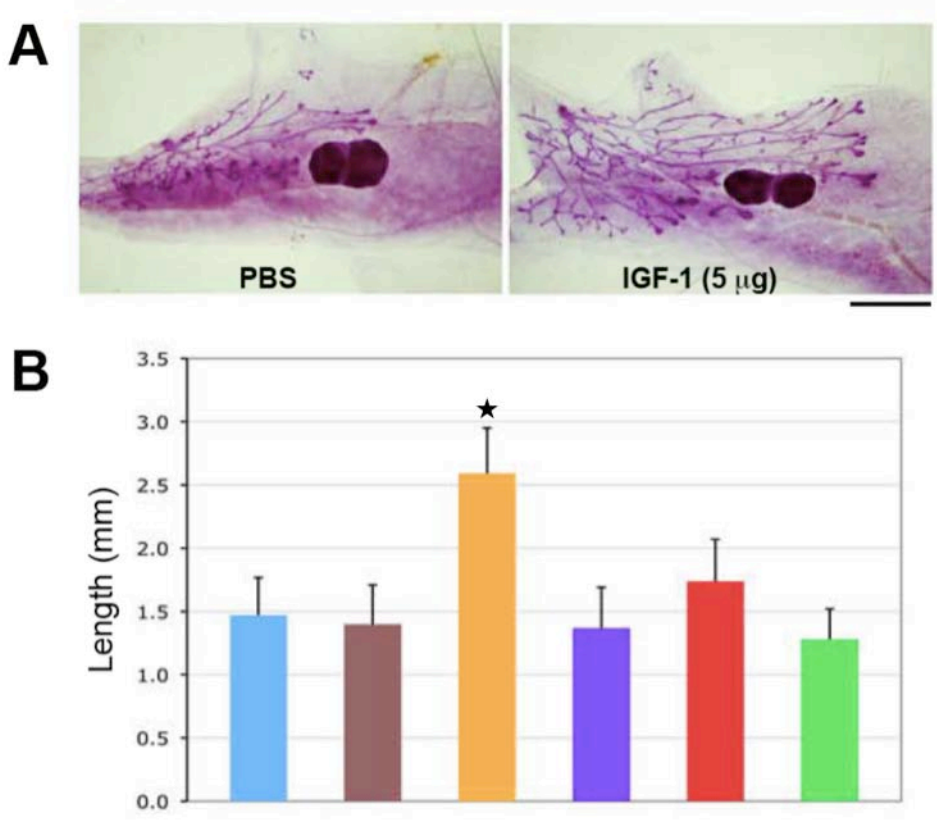

Figure 5. (A) Whole mount carmine alum staining showing the extent of ductal elongation from the mammary glands of C57BL/6J female offspring born to a mother that were injected with PBS (left panel) or 5 µg of IGF-1 (right panel). (B) Histograms showing the extent of ductal elongation in mammary glands of C57BL/6J female offspring born to mothers that were injected with PBS (blue, n = 15) or IGF-1 consisting of 2.5 µg (brown, n = 9), 5 µg (yellow, n = 12), 10 µg (purple, n = 10), 20 µg (red, n = 10), or 50 µg (green, n = 11). Error bars represent standard error of the mean (SEM). * denotes significance of $p < 0.05$ by *t*-test when compared to PBS controls.

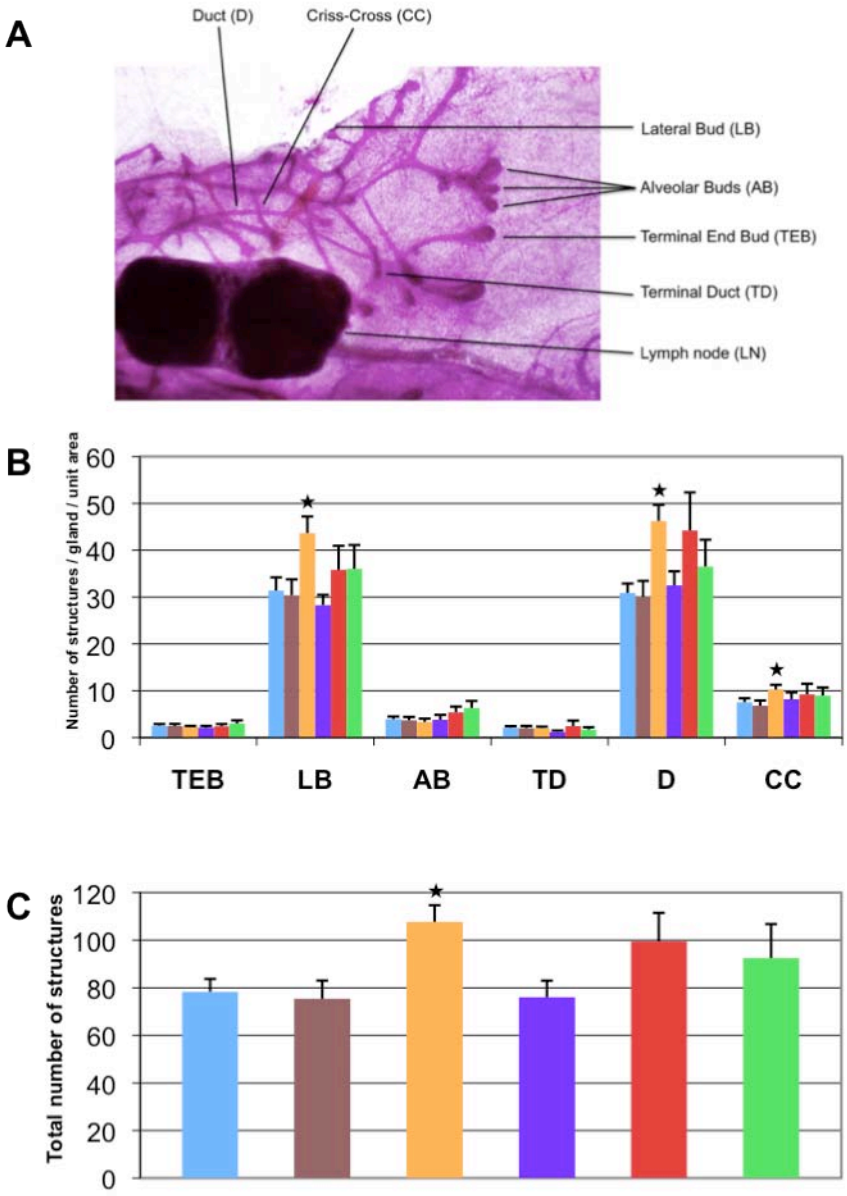

Figure 6. (A) Whole mount carmine alum staining showing mammary structures used in the morphometric analysis. (B) Histograms showing the number of terminal end buds (TEB), lateral buds (LB), alveolar buds (AB), terminal ducts (TD), ducts (D), and criss-crosses (CC) from mammary glands of C57BL/6J pups born to mothers that were injected with PBS (blue, n = 15) or IGF-1 consisting of 2.5 µg (brown, n = 9), 5 µg (yellow, n = 12), 10 µg (purple, n = 10), 20 µg (red, n = 10), or 50 µg (green, n = 11). (C) Histograms showing the sum of all the mammary structures per unit area of each mammary gland as a measure of breast density. Error bars represent standard error of the mean (SEM). ⋆ denotes significance of $p < 0.05$ by *t*-test when compared to PBS controls.

Although unlikely, there was concern that the observed effects from the exogenous IGF-1 treatment might be due to the simultaneous presence of endogenous levels of IGF-1 in the wild type mice. To confirm our results and to circumvent the effects of endogenous IGF-1 levels, we tested the effects of *in utero* levels of IGF-1 using a strain of mice homozygous for the $Igf1^{tm2Ts}$ mutation in which a targeting construct consisting of the neomycin-resistance gene, the thymidine kinase gene, and vector sequences were inserted just upstream of the 5' end of exon 3 of the *Igf1* gene. These IGF-1 deficient ($IGF\text{-}1^{m/m}$) mice are fertile and viable but with body weights 60 to 65% and serum IGF-1 levels 30% that of wild type (Lembo et al., 1996). We were successful in the breeding of homozygous $Igf1^{m/m}$ mice (Task b) and using these mice in the study (Task d). We are currently analyzing the data from these IGF-1 deficient mice (Task e) and preliminary analysis using these IGF-1 deficient mice continued to display similar effects in body weights, breast stem cell numbers, and breast

density measurements as seen in wild type mice (Figure 7). A manuscript reporting the findings of this study is ready for submission (Task e).

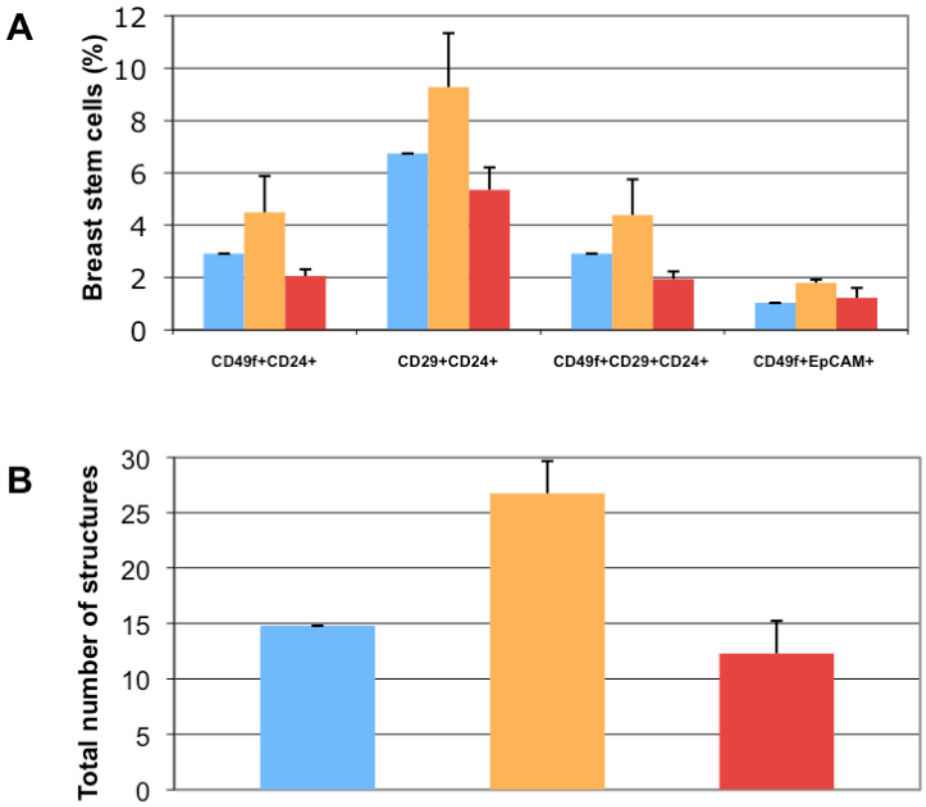

Figure 7. Histograms showing the preliminary analysis of (A) the percentages of putative breast stem cell populations and (B) the sum of all the mammary structures per unit area of each mammary gland as a measure of breast density from the mammary glands of IGF-$1^{m/m}$ offspring born to mothers that were injected with PBS (blue, n = 1) or IGF-1 consisting of 5 µg (yellow, n = 2) and 20 µg (red, n = 3). Error bars represent standard error of the mean (SEM).

KEY RESEARCH ACCOMPLISHMENTS

• Putative populations of breast stem/progenitor cells such as the $CD49f^{+}CD24^{+}$, $CD29^{+}CD24^{+}$, $CD49f^{+}CD29^{+}CD24^{+}$, and $CD49f^{+}EpCAM^{+}$ cells were identified from dissociated mammary gland cells by flow cytometry.
• Breast density was successfully assessed as the sum of all the mammary structures per unit area per mammary gland.
• Coverslipping using Permount of whole mount carmine alum stained mammary glands preserved the histological staining indefinitely.
• Daily intraperitoneal injections of 5 µg total IGF-1 into pregnant C57BL/6J or IGF-$1^{m/m}$ mice from gestational days 10 to 18 were sufficient to significantly increase the body weights, number of breast stem cells, and breast density in the prepubescent female offspring.
• Prenatal exposure to 5 µg of total IGF-1 influences postnatal growth (but not birth weight) as reflected in significantly heavier body weights at post-natal day 3, 7, and even up to 21, when compared to PBS controls.
• Prenatal exposure to 5 µg of total IGF-1 resulted in a significant increase in the $CD49f^{+}CD24^{+}$ and $CD49f^{+}CD29^{+}CD24^{+}$ breast stem/progenitor cell subpopulations when compared to PBS controls.
• Prenatal exposure to 5 µg of total IGF-1 resulted in longer duct elongation when compared to PBS controls.
• Prenatal exposure to 5 µg of IGF-1 resulted the densest mammary gland as assessed by the sum of all the mammary structures per unit area per mammary gland.

REPORTABLE OUTCOMES

• An abstract on the findings of this study was presented as a poster at the Era of Hope 2011 meeting (Chang et al., 2011 – see References #2 and Appendix 2).

• A paper on the prenatal modulation of breast stem cells and breast density by insulin-like growth factor-1 is being prepared for submission for publication.
• An *in utero* animal model of IGF-1 modulation is established in this study.

CONCLUSION

An *in utero* animal model of IGF-1 modulation is established in this study. Daily intraperitoneal injections of 5 μg total IGF-1 into pregnant wild type C57BL/6J or IGF-1-deficient mice from gestational days 10 to 18 are sufficient to significantly increase the body weights, number of breast stem cells, and breast density in the prepubescent female offspring. Notably, this experimental data provide a direct evidence for a prenatal modulation of breast density in the offspring by IGF-1 and confirms similar associations observed in epidemiological studies. In other words, the findings demonstrate that *in utero* exposures to low levels of mitogens may be sufficient to influence cancer risk in the adult life. More importantly, since high breast density is a significant risk factor of breast cancer, the findings of this study support a paradigm for a prenatal mechanism affecting breast cancer risk involving mammary gland-specific stem cells. Hence, exploring prenatal mechanisms for the regulation of putative breast stem/progenitor cells via the IGF-1 axis could further our understanding of breast carcinogenesis and potentially lead to novel strategies for preventing breast cancer in the adult life. The applicability of this research would be the identification of potential breast cancer-susceptibility genes that are sensitive to *in utero* IGF-1 exposures. More broadly, the findings on the 'adverse' effects of IGF-1 could inform future human health studies in the characterization of labile genes due to prenatal exposures of other mitogens including environmental endocrine disruptors.

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APPENDICES

Appendix 1. List of personnel

Chung-Cheng Hsieh
Hoi Pang Low
Chien-I Chang
Li Qiu

Appendix 2. Bibliography

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Poster P28-3

BC074347-2705

PRENATAL MODULATION OF BREAST STEM CELLS AND BREAST DENSITY BY INSULIN-LIKE GROWTH FACTOR 1

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Background: The biological determinants of breast density, a strong predictor of human breast cancer risk, can be influenced by prenatal exposure to mitogens. We postulate that breast density is determined in large part by the number and activity of breast stem cells that arise during the in utero/perinatal period: the larger the breast stem cell pool, the higher the breast density. We also hypothesize that the number of such breast stem/progenitor cells is in turn correlated with in utero levels of mitogens. Therefore, the objective of the study was to determine the extent to which in utero levels of insulin-like growth factor 1 (IGF-1) would affect the birth/body weight, levels of breast stem/progenitor cells, and breast density of prepubescent mice.

Methods: We administered intraperitoneally different doses of IGF-1 to pregnant C57BL/6J mice from embryonic Days 10–18. The body weights of the offspring were monitored regularly and prepubescent females were sacrificed at approximately 4 weeks of age. The left fourth inguinal mammary glands were dissected out for flow cytometric and histological analyses.

Results: We found that administration of 5 and 20 µg of IGF-1 resulted in significantly heavier body weights of the pups at postnatal Day 3 ($p = 0.005$ and 0.02, respectively), Day 7 ($p = 0.0001$ and $p = 0.002$, respectively), and even up to Day 21 ($p = 0.0001$ and $p = 0.005$, respectively) when compared to phosphate buffered saline (PBS) controls, but not at birth. Flow cytometric analysis of dissociated cells from the left fourth inguinal mammary gland displayed a similar positive association where treatment with 5 µg of IGF-1 resulted in the most number of putative breast stem/progenitor cells ($p = 0.01$ and 0.01 for the $CD49f^+CD24^+$ and $CD49f^+CD29^+CD24^+$ populations, respectively), followed by 20 µg IGF-1 ($p = 0.07$ and 0.04, respectively) when compared to PBS controls. There was no significant difference in the number of $CD49f^+CD29^+$, $CD49f^+EpCAM^+$, and $CD29^+CD24^+$ cells for all treatment groups when compared to controls. Histological analysis of whole mount carmine alum staining of the contralateral fourth inguinal gland suggested that breast density, as assessed by the sum of the different mammary gland structures per unit area, was highest at the lowest dose of 5 µg IGF-1 followed by the treatment with 20 µg of IGF-1. Interestingly, the highest dose of IGF-1 tested (50 mg) consistently resulted in inverse associations with body weight, levels of breast stem/progenitor cells, and breast density when compared to the lower doses, although it was not significantly different from the control animals.

Conclusions: These findings provide direct evidence for a prenatal modulation of breast density possibly involving breast stem/progenitor cell levels in the offspring by low levels of IGF-1. The quantitation of breast stem/progenitor cell markers in biopsy samples or umbilical cord blood levels of IGF-1 could be used for the identification of high-risk individuals. Exploring prenatal mechanisms for the regulation of putative breast stem/progenitor cells via the IGF-1 axis could further our understanding of breast carcinogenesis and potentially lead to strategies for preventing breast cancer in the adult life.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-08-1-0456.